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Stimulation of Cell Invasion and Migration by Alcohol in Breast Cancer Cells

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Increasing epidemiological studies suggest that alcohol consumption confers a high risk for development of breast cancer. In this study, we found that biologically relevant concentrations of alcohol elicited a significant stimulation of cell adhesion, migration, and invasion in MCF-7 human breast cancer cells. Moreover, the promotion of invasion and migration potential by alcohol was associated with the significant decrease of E-cadherin, α, β, and γ three major catenin, and BRCA1 expression. In addition, an enhanced expression of BRCA1 significantly blocked alcohol-stimulated cell invasion. Thus, our present study suggests that alcohol as a breast cancer risk factor plays an important role not only in carcinogenesis, but also in promotion of cell invasion and migration.

Epidemiological studies have documented a close relationship between alcohol consumption and breast cancer rates in women (reviewed in 1–3). In a pooled analysis of six prospective cohort studies that examined dietary factors in breast cancer, increasing alcohol intake linearly correlated with the breast cancer risk (4). Alcohol consumption is one of the defined environmental risk factors for breast cancer, although the mechanism of alcohol-induced carcinogenesis needs to be understood (5, 6).

Breast cancer is the most common cancer in women. Although majority of the early-stage breast cancers are not life threatening, a small amount of primary cancer cells will progress to metastatic breast cancer which gives rise to a devastating and largely incurable disease and is mayor cause of the death. It have been known that the invasive behavior of primary breast tumor cells and their potential to metastasize to the other organs are complex biological processes that begins with the detachment of cancer cells from the original tumor mass, the attachment to extracellular matrix binding sites, the degradation of the matrixes, and the invasion into the adjacent tissue and the surrounding blood and lymphatic vessels. A small proportion of these detached breast cancer cells stops in distant organs by the attachment to the basement membrane of these organs, by the secretion of enzymes that cause a degradation of this membrane barrier, and by the migration of primary cancer cells into the target tissue, and finally forms secondary breast cancer (metastasis of breast cancer). In this study, we examined the effect of alcohol on the migration and invasion behaviors of breast cancer cells.

Abbreviations used: BRCA1, breast cancer susceptibility gene 1; MTT, tetrazolium salt [3-(4,5-dimethylthuazole-2-yl)-2,5 diphenyl tetrazolium bromide.

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MATERIALS AND METHODS

Cell culture and BRCA1 plasmid. MCF-7 human breast cancer cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and maintained as monolayer cultures in D-MEM supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 unit/ml penicillin G (BioWhittaker, Walkersville, MD). Before each experiment, subconfluent monolayers of MCF-7 cells were resuspended in D-MEM and passed repeatedly through a 25-gauge needle to produce a single cell suspension. Cell number and viability were determined by staining a small volume of cell suspension with 0.4% trypan blue saline solution and examining the cells using a hemocytometer. Full-length BRCA1 cDNA was encoded in a pcDNA3 expression plasmid as described previously (7).

MTT survival assay. Cytotoxicity of alcohol was measured by a MTT survival assay as described previously (7). MTT assay is a commonly used method in evaluation of cell survival, based on the ability of viable cells to convert MTT, a soluble tetrazolium salt [3-(4,5-dimethylthuazole-2-yl)-2,5 diphenyl tetrazolium bromide], into an insoluble formazan precipitate, which is quantitated by spec-

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trophotometry following solubilization in dimethyl sulfoxide (DMSO). Briefly, MCF-7 cells untreated and treated with alcohol in 96-well tissue culture dishes were incubated with MTT (2 μg/ml) for 4 h. The cells were then solubilized in 125 μl of DMSO and absorbance readings were taken using a Dynatech 96-well spectrophotometer. The amount of MTT dye reduction was calculated based on the difference between absorbance at 570 and 630 nm. The cell viability in treated cells was expressed as the amount of dye reduction relative to that of untreated control cells. The wells which contained only medium and 10 μl of MTT were used as blanks for the plate reader. Four sets of experiments were performed in 10 wells for each treatment. MTT was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared in sterile deionized water and stored at 4°C prior to use.

Cell adhesion assay. The 24-well tissue culture dishes were coated with 25 μg/well Matrigel basement membrane extract (Collaborative Biomedical Products, Bedford, MA) and left to air-dry. The concentration of Matrigel used in the experiments was determined in a previous study in which we varied the amount of reconstituted basement membrane placed on the dishes (data not shown). Aliquots (3×10^5 cells/ml) of the cell suspension containing 80 mM alcohol were seeded into the Matrigel-coated wells and incubated for 10, 30, 60, 90, 120, or 150 min at 37°C in 5% CO_2. At the end of these periods, the cells were washed gently three times with PBS to remove the unattached cells, and the attached cells were then harvested. Both unattached and unattached cells were counted. Experiments were independently performed at least three times.

Invasion assay. Invasion assay was carried out using a modified Boyden chamber assay as described previously (8). Briefly, the surfaces of filter (0.8 μm pore size) were coated with a uniform thickness of 25 μg Matrigel for 60 min at room temperature. Uniformity of the coating was checked by Coomassie blue staining and low-power microscopic observation. The lower chamber was filled with 10% serum medium. Fibronectin (16 μg/chamber) as the chemoattractant was added to the lower chamber. Cells (1×10^5 cells/ml) resuspended in the medium containing 40 or 80 mM alcohol were carefully transferred onto the upper surface of filters in the chamber. After 24 h incubation, the filter was gently removed from the chamber, the cells on the upper surface were removed by wiping with a cotton swab, and the cells that invaded the Matrigel and attached to the lower surface of the filter were fixed, stained with H & E, and counted in 15 randomly selected microscopic fields (×400) per filter. Experiments were performed independently at least three times.

Cell migration assay. MCF-7 cell motility was assessed using a scratch wound assay (9). The cells (2.5×10^5 cells) were seeded into six-well tissue culture dishes and cultured in medium containing 10% FCS to confluent cell monolayers, which were then carefully wounded using sterile pipette tips and any cellular debris was removed by washing with PBS. Fibronectin (16 μg/ml) was added to the culture. The wounded monolayers were then incubated in 10% FCS D-MEM containing 40 or 80 mM alcohol for 24 h and photographed under a phase contrast microscope. The experiments were repeated in quadruplicate wells at least three times (×200).

Protein analysis. Expression of BRCA1, E-cadherin, and α-, β-, and γ-catenin protein was assayed using a Western blot assay as described previously (7). Untreated and treated cells were lysed in a PBS buffer containing 1% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride, 10 mM sodium fluoride, 1 mM sodium *o*-orthovanadate, and 5 mM sodium pyrophosphate. Fifty micrograms of total protein lysates was electrophoresed on 6 or 10% SDS–polyacrylamide gels and transferred to membranes by electroblotting. The membranes were incubated with a recommended dilution of primary antibodies against BRCA1 (C-20, Santa Cruz, Hercules, CA), E-cadherin, α, β, and γ-catenin (Transduction Laboratories, Lexington, KY), and then incubated with secondary antibodies after being extensively washed. Antibody reaction was revealed using an enhanced chemiluminescence detection system (Amersham Life Science, Arlington Heights, IL) as instructed by the manufacturer. Equal protein loading and the protein transfer were confirmed by immunoblotting for determination of α-Actin using polyclonal α-actin antibody (I-19, Santa Cruz) in the same Western blots. A colored marker (Bio-Rad Laboratories, Hercules, CA) was used as a molecular size standard.

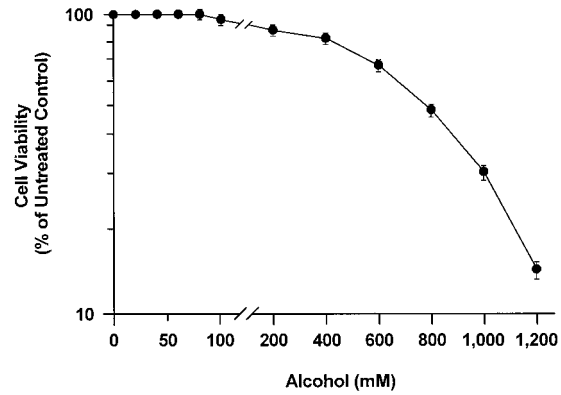

FIG. 1. Cytotoxicity of alcohol in MCF-7 human breast cancer cells. Exponentially growing cells in 96-well tissue culture dishes were exposed to alcohol in serum-free medium for 24 h and assayed for cell viability by the MTT dye conversion assay. Values are mean ranges of the average cell viability for three independent experiments.

RESULTS AND DISCUSSION

To determine the cytotoxicity of alcohol in MCF-7 human breast cancer cells, exponentially growing cells were exposed to various concentrations of alcohol for 24 h and then subjected to the MTT assay as described under Materials and Methods. As shown Fig. 1, alcohol exhibited a dose-dependent cytotoxicity in MCF-7 cells. IC_{50}, defined as the alcohol concentration results in 50% loss of cell viability relative to untreated cells, was approximately 760 mM. However, no any cytotoxicity of alcohol was observed below 100 mM. Thus, 40 and 80 mM concentrations were chosen to perform further study.

Tumor metastasis comprises of multiple steps and thus tumor cells are required to express a variety of properties including altered adhesiveness, increased motility and invasive capacity to complete the metastatic process. Adhesion to the basement membrane is the first step in the metastasis cascade. To examine the effect of alcohol on cell adhesion, human breast cancer MCF-7 cells were plated to the six-well tissue culture dishes coated with Matrigel. A significantly increased adhesion of MCF-7 cells to the Matrigel was observed in the medium containing 80 mM alcohol compared to the medium containing no alcohol (Fig. 2a). For example, at 60 min following plating of cells, only 65% cells attached on the plates in the absence of alcohol, whereas approximately 95% cells attached in the pres-

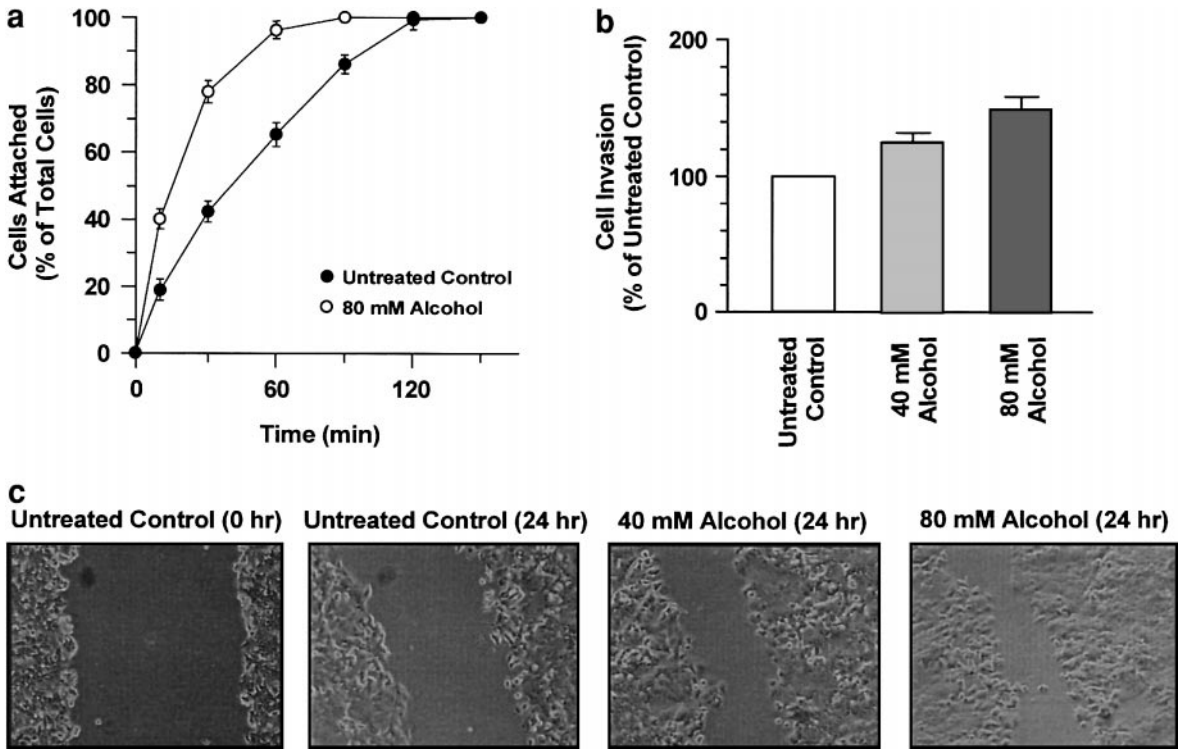

FIG. 2. Alcohol promotes adhesion, invasiveness and migration in human breast cancer MCF-7 cells. (a) Adhesion assay. MCF-7 cells (3×10^5) suspended in D-MEM containing 80 mM alcohol were plated in each well of 24-well Matrigel (50 μg)-coated plates, the medium was gently removed and attached cells were counted at the indicated time following plated. Data are expressed as mean ± SE of three independent experiments. (b) Invasion assay. Subconfluent growing MCF-7 cells were trypsinized and counted, resuspended in 2.5% serum medium containing alcohol (40 or 80 mM) and transferred into the upper compartment of the modified Boyden chambers (1×10^5 cells/chamber). Fibronectin (16 μg/chamber) as a chemoattracter was added to the lower compartment. At 24 h later, invaded cells that attached to the lower surface of the filter coated with Matrigel (25 μg/chamber) were counted in 15 randomly selected microscopic fields (×200) per filter. Data are expressed as the percentage of the control response (mean ± SE). (c) Wound migration assay. Confluent MCF-7 cells cultured in six-well dishes were carefully wounded using sterile pipette tips ($T = 0$ h) and then recultured in the medium with or without alcohol (40 or 80 mM). At 24 h after wounded, the cells were photographed under a phase contrast microscope.

ence of alcohol ($P < 0.05$, Student's t test). These results indicate that alcohol promotes cell adhesion to the Matrigel.

A critical event in tumor invasion and metastasis is the ability of tumor cells to invade through the extracellular matrix, allowing tumor cells to move beyond the confines of the primary tumor environment. To examine the effect of alcohol on cell invasion, a modified Boyden chamber assay was carried out to determine the ability of MCF-7 cells to invade through biological matrices *in vitro*. As shown in Fig. 2b, when 40 or 80 mM alcohol was present in the upper chamber, the cells to invade through the filter coated with Matrigel was increased by approximately 23% and 52% compared to the untreated control, respectively.

To examine whether the alcohol inhibition of cell invasion was associated with its suppression on cell motility, the effect of alcohol on the migration capability of MCF-7 cells was also analyzed using a scratch wound assay. Confluent monolayers of MCF-7 cells were scratch wounded with sterile pipette tips and post-incubated for a further 24 h in the absence and the presence of alcohol (40 or 80 mM). Cell flattening and spreading along the edges of the wound was significantly enhanced in the presence of alcohol compared to the untreated control (Fig. 2c). Similar stimulation of cell invasion and migration was also observed in another human breast cancer cell line, T-47D (data not shown). Taken together, these results indicate that biologically relevant concentrations of alcohol significantly promoted the cell adhesion, invasion and migration in human breast cancer cells.

To ascertain whether the stimulation of *in vitro* adhesion, invasiveness and migration by alcohol was accompanied by any changes in E-cadherin/catenin cell-cell adhesion molecules, we also analyzed the protein expression of E-cadherin and three major (α, β and γ) catenins by a Western blot assay. As illustrated in Fig. 3a, 24-h treatment of alcohol caused a dose-dependent decrease in E-cadherin protein expression. Also, a downregulated expression of α, β, and γ-catenin protein occurred in parallel with the decrease of

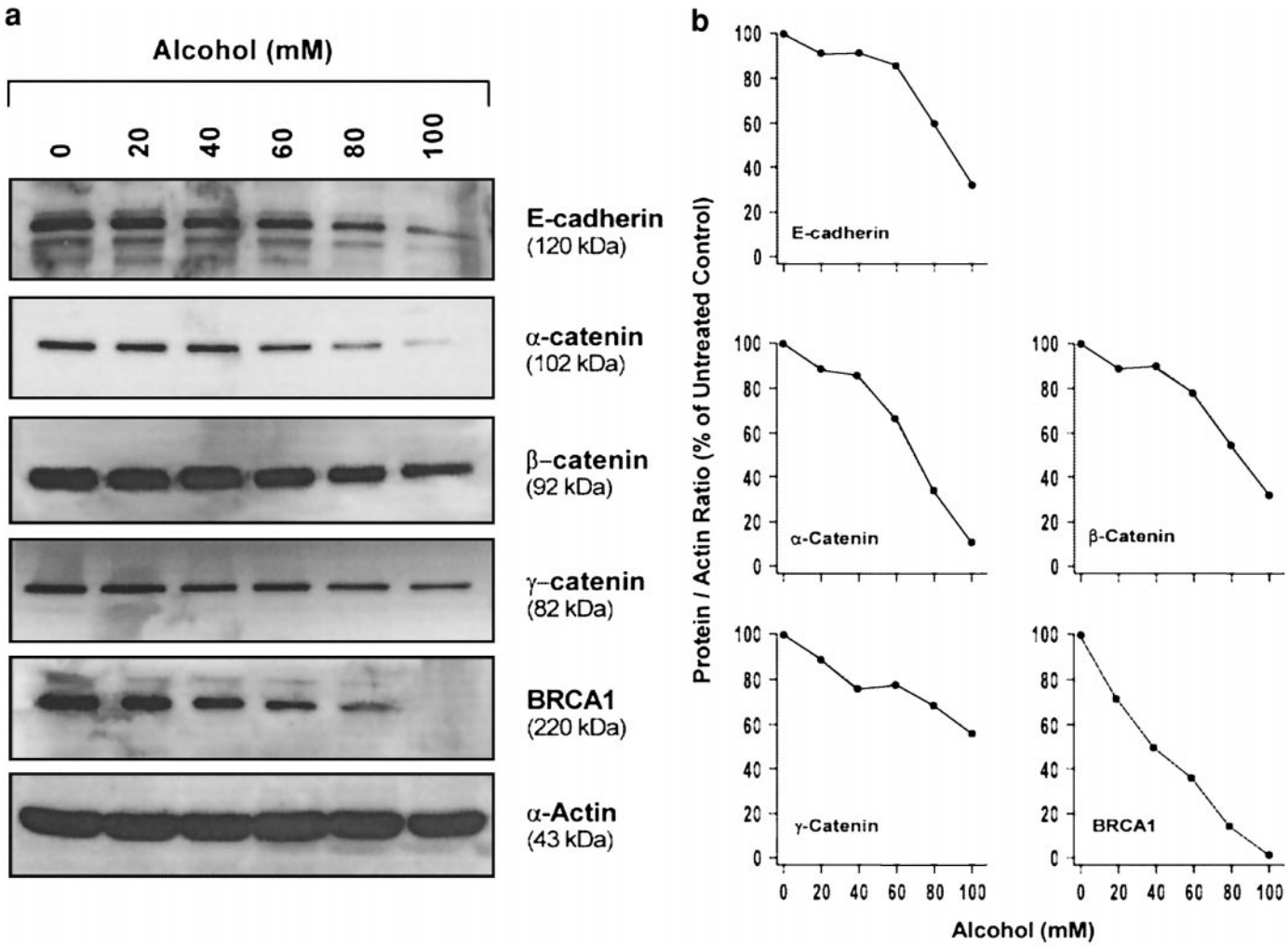

FIG. 3. Alcohol downregulates expression of E-cadherin, α-, β-, and γ-catenin and BRCA1 protein. (a) Subconfluent growing MCF-7 cells ware treated with alcohol at the indicated doses for 24 h and harvested for Western blotting as described previously (7). Equal aliquots of total protein (50 μg per lane) were analyzed on 6 or 12% SDS–polyacrylamide gels and blotted to detect E-cadherin, α-, β-, and γ-catenin, BRCA1 and α-actin (as a control for protein loading and transfer) protein expression. (b) Protein bands in panel *a* were quantitated by densitometry and expressed relative to the α-actin bands.

E-cadherin following alcohol treatment. The bands of these proteins were quantitated by densitometry and presented as the percentage of untreated control in Fig. 2b. E-cadherin is a transmembrane glycoprotein responsible for the maintenance of cell adhesion and tissue integrity by complex with three major cytoplasmic catenins (α, β and γ) (10). E-cadherin/catenin complexes have been identified as key players in the progression of a variety of human carcinoma cells, including breast cancer, and the acquisition of an invasive phenotype (11, 12). Moreover, one of these E-cadherin/catenin proteins was downregulated, the function of the others in suppressing metastasis is also altered (13). Disruption of the E-cadherin/catenin complex, primarily due to loss or decreased expression of E-cadherin, is correlated with the progression of breast cancer by increasing proliferation, invasion and distant metastasis (14–17). Restoration of E-cadherin function was able to dramatically suppress invasive phenotype (18). Additionally, it was also found that many agents, like tamoxifen (18), melatonin (19), and indole-3-carbinol (20), exhibited their anti-invasion and migration capabilities in breast cancer by increasing the expression of E-cadherin/catenin complexes. Although a defective E-cadherin/catenin complex may not be the sole factor that determines the invasive phenotype of breast cancer cells, it is proposed, based on our present observations, that the down-regulation of E-cadherin/catenin complexes is an important mechanism for the enhancement of invasion and migration in breast cancer cells exposed to alcohol.

Mutations of breast cancer susceptibility gene 1 (*BRCA1*) confer a high risk for breast cancer (21, 22). Increasing evidence indicates that invasive breast can-

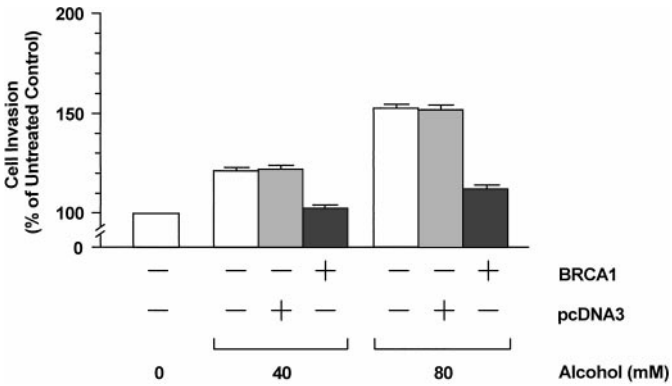

FIG. 4. Overexpression of BRCA1 decreases stimulation of cell invasion and migration by alcohol. Exponentially growing MCF-7 cells cultured on 100-mm tissue culture dishes were transfected overnight with the pcDNA3 expression plasmid encoding full-length BRCA1 cDNA and the "empty" control pcDNA3 plasmid (as a control) with the help of a transfection reagent, lipofactin (Gibco-BRL, Gaithersburg, MD) and then incubated for a further 24 h to maximize BRCA1 expression before the assay of cell invasion which was performed as described in Fig. 2b. Transfection efficiency was evaluated by determination of β-gal activity via co-transfection with β-gal vectors. Data are expressed as percentages of the control without transfection and alcohol treatment (means ± SE) from two independent experiments.

cers show a decreased BRCA1 mRNA expression and a loss of BRCA1 immunochemical staining compared to non-invasive cancers and benign tissues (23–29). A recent animal study also found that loss of BRCA1 expression is correlated with an increased likelihood of distant metastasis (30). In addition, our recent studies showed that indole-3-carbinol, a phytochemical chemoprevention compound, causes an induction of *BRCA1* gene expression in the suppression of breast cancer invasion and metastasis (20). These findings suggest a potential role for BRCA1 in the mediation of breast cancer invasion and metastasis. In the present study, we found that alcohol down-regulated the expression of the BRCA1 protein in a dose-dependent manner (Fig. 3). Moreover, an increased expression of BRCA1, by transient transfection with pcDNA3 vector encoding wild-type full–length BRCA1 cDNA, significantly abolished the promotion of MCF-7 cell invasion due to alcohol treatment, approximately 72 and 82% reduction at 40 and 80 mM, respectively (Fig. 4). The control pcDNA3 vector did not give rise to any effects on the alcohol capability in stimulating the invasion of MCF-7 cells. These results indicate that BRCA1 is an important mediator in the promotion of breast cancer cell invasion and migration by alcohol.

In summary, we provided *in vitro* evidences for first time that alcohol at biologically relevant concentrations promotes the breast cancer cell adhesion, migration and invasion through modulating E-cadherin, α, β, γ-catenin and BRCA1 expression. Therefore, our present study indicates that alcohol acts as a breast cancer risk factor not only in carcinogenesis, but also in the promotion of breast cancer invasion and metastasis.

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